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# HPLC Photodiode Array Analysis of Rifampin in Actinobacterial Secondary Metabolites

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## Abstract

In this study, the potential of rifampin production and characterize actinobacterial secondary metabolites using High-performance liquid chromatography (HPLC) analysis. A total of 9 samples were collected from different depths of Desert soils in Al-Anbar Governorate, Iraq, employing the spread plate technique on various agar media. Through 16s rRNA analysis, GenBank, and phylogenetic analysis, three rare actinobacterial strains were identified. To compare with crude extracts, rifampin was used as a standard for qualitative and quantitative HPLC analysis, based on rotation time. Following secondary metabolite extraction, HPLC analysis was conducted to characterize different extracts. The HPLC pattern analysis revealed that isolates AR7, AR21, and AR42 exhibited potential as rifampin producers. Additionally, secondary screening of crude extracts against six pathogenic microbes demonstrated varying degrees of inhibition zones. These findings underscore the significance of actinobacterial isolates in producing antibiotic compounds. Previous studies have established that actinobacteria harbor numerous compounds with antimicrobial, analgesic, anti-inflammatory, and antioxidant properties.

**Keywords: Desert soil, Actinobacterial, Antimicrobial activity, Phylogenetic analysis, Metabolite extraction**

## 1. Introduction.

Rifampin, also known as Rifampicin, is an antibiotic derived from the actinomycete *Amycolatopsis rifamycinica* (Surette and Wright, 2021). It is widely used for the treatment of tuberculosis (TB) (AlMatar et al., 2019). Rifampin exerts its antimicrobial effects by inhibiting the activity of DNA-dependent RNA polymerase in pathogenic organisms, thereby blocking the initiation of RNA synthesis (Das, 2022). This bactericidal compound is effective against both intracellular and extracellular pathogens. Discovered in 1965, Rifampicin is included in the World Health Organization's List of Essential Medicines due to its critical role in healthcare (Vilchèze et al., 2018). Actinobacterial, including *Amycolatopsis rifamycinica*, are important producers of secondary metabolites with significant implications for human health (Sood et al., 2024). Actinobacteria, in particular, have been recognized for their ability to generate unique secondary metabolites and exhibit diverse biological activities, making them valuable resources in medical and pharmaceutical industries (Genilloud, 2017). Actinbacterial have been a focal point of research due to the identification and isolation of natural compounds that contribute to the development of drugs for human, veterinary, and agricultural purposes (Takahashi & Nakashima, 2018). Therefore, exploring actinomycetes for the production of secondary metabolites and bioactive compounds remains a crucial objective (Subramani & Sipkema, 2019). In the present study, our main aim was to screen, isolate, and characterize potential antibiotic-producing strains of actinobacteria, with a specific focus on Rifampin, using HPLC profiling of actinobacteria isolated from Desert soil in Al-Anbar. Additionally, we conducted antimicrobial screening of crude extracts, a significant aspect of our research. Streptomycetes and related actinomycetes continue to be valuable sources of novel secondary metabolites with diverse biological activities (Parra et al., 2023). Such compounds may find applications as anti-infectives, anticancer agents, or other pharmaceutically useful substances, highlighting the ongoing importance of actinomycete research and isolation to discover novel compounds.

## 2. Materials and Methods.

### 2.1 Collection of Samples.

Soil samples were collected from the Al-Anbar Desert at three distinct depths: surface, 30cm, and 100cm. These samples were carefully placed in sterile plastic bags to maintain their integrity. To preserve their quality, a total of eighteen samples were stored on ice during transportation to the laboratory. Upon arrival, the samples were stored at a temperature of 4 °C for subsequent analysis.

### 2.2 Actinobacterial from Soil.

The isolation of rare actinobacteria from desert soil was primarily conducted using four different media. The media employed were ISP Medium No.2 (Shirling & Gottlieb, 1966), Starch M-protein agar (Küster & Williams, 1964), Bennett's agar (Jones, 1949), and Actinomyces agar (Kornman & Loesche, 1978). The soil dilution plate method was employed for the isolation process. To inhibit fungal growth and counteract gram-negative bacteria, cycloheximide and nalidixic acid were added to the media at a final concentration of 25 µg/ml (Donald et al., 2022).

### 2.3 Primary Screening of Actinomycetes Isolates.

Primary screening, an agar plug method was employed, following the procedure outlined by Idris et al. (2017). Sterile cork borers were used to create plugs of approximately 0.5 cm in diameter from the actinomycete culture plates that had been incubated at 30°C for 14 days. These plugs were then placed onto plates that were previously seeded with various test organisms. The plates were subsequently incubated at 37°C (Ramasamy, 2022). After 24 hours of incubation, the diameter of the inhibition zones was measured as described by Salleh et al. (2020). Strains exhibiting significant antimicrobial activity during the screening were selected for further molecular identification.

### 2.4 Phylogenetic analysis.

The actinobacteria were cultured on ISP No.2 medium agar (Shirling & Gottlieb, 1966) for a duration of two weeks at a temperature of 30°C. Genomic DNA extraction was performed using the Genomic DNA Purification Kit (Thermoscientific, UK) following the provided protocol. To amplify the 16S rRNA gene fragment, universal primers 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1525R (5' AAA GGA GGT GAT CCA GCC 3') were used in accordance with the method outlined by Lane (1991). Prior to sequencing, the PCR product underwent purification through MyTACG Sdn Bhd. The obtained sequences were edited using BioEdit version 7.2.5 (Rassem et al., 2018), with ABI format files as the input. The edited sequences were then converted into FASTA format files. The nearest matches for each edited sequence were determined using the Basic Local Alignment Search Tool (BLAST), and pairwise sequence similarities were calculated using the EzTaxon-e web server (http://eztaxon-e.ezbiocloud.net/; Idris et al., 2018). The verified and adjusted alignment was utilized to construct a phylogenetic tree based on the neighbor-joining method, employing MEGA 7.0 Analysis (Idris et al., 2018). The confidence level of the associations was determined based on 1,000 bootstraps (Idris et al., 2017).

### 2.5 Extraction of Secondary Metabolite.

The actinobacterial strains were streaked onto ISP No.2 medium agar and incubated at a temperature of 30°C for a duration of one week. Afterward, the colonies were scraped from each plate and transferred into 1000 mL of ISP No.2 broth medium. The broth cultures were placed in a rotary shaker at 125 rpm and 30°C for a period of two weeks, as described by Rodríguez et al. (2018). Following incubation, centrifugation was performed at 12,000 rpm for 30 minutes to separate the cells from the culture.To extract the extracellular crude metabolites, a liquid-to-liquid extraction method was employed. An equal volume of methanol was used, following the procedure described by Matsumoto and Takahashi (2017). The crude extracts of secondary metabolites were obtained by drying the preparations using a Rotary Evaporator (rotovap) at a temperature of 40°C, as outlined by Rodríguez et al. (2018).

### 2.6 Secondary Screening of Metabolite Extracts.

The screening of actinobacterial crude metabolites was conducted using the agar diffusion method, as described by Biemer (1973) and RAMLI (2018), against a panel of microorganisms. In parallel, fresh Muller-Hinton broth was inoculated with pathogenic microorganisms and incubated at a temperature of 37°C with shaking at 100 rpm for one day. Sterile paper discs with a diameter of 6 mm, which had been previously impregnated with the crude metabolites, were placed on agar plates containing 100 µl of the pathogenic microorganisms. The plates were then incubated at 37°C. After one day, the antimicrobial activity was evaluated by measuring the diameter of the translucent inhibition zones formed around the discs. As positive controls, antibiotic discs containing Ciprofloxacin (CIP, 10 µg/disc), Vancomycin (VA, 30 µg/disc), and Tetracycline (TE, 30 µg/disc) were used, whereas a disc containing only methanol served as the negative control, following the protocol outlined by Jegan et al. (2019).

### 2.7 HPLC analysis.

To analyses the dried crude extracts from three species, they were dissolved in 100 ml of methanol to achieve a concentration of 100 µg/ml. A 100 µg/ml Rifampicin standard solution was prepared by dissolving 0.1 grams of Rifampicin in deionized water and diluting it to 1000 millilitres. Subsequently, a 100 µg/ml Rifampicin standard was obtained by diluting 10.00 millilitres of the stock solution to 100 mL using deionized water, following the procedure outlined by Rasheduzzaman et al. (2018). The analysis of the crude extracts was performed using an HPLC system from Agilent (Germany), equipped with an Agilent column measuring 15 cm x 0.2 µm x 0.25 µm ID and a DAD detector set at a wavelength of 254 nm. The mobile phase consisted of 20% methanol and 80% water, with a total run time of 10 minutes, as described by Mathur et al. (2015). For each sample, a 3 µl injection volume was used, and the elution flow rate was set at 500 µl/min.

## 3. Results and Discussion.

### 3.1 Primary Screening of Actinobacterial Isolates.

The initial screening of the actinobacterial isolates revealed significant antimicrobial activity against a majority of the pathogenic microorganisms. However, in this study, it was observed that S. aureus and P. aeruginosa exhibited higher resistance to the isolates compared to the other tested microorganisms (Table 1).

Table 1. Inhibition zones for actinobacterial isolates when tested against a panel of microorganisms

| | Tested microorganisms | | | | | |
|---|---|---|---|---|---|---|
| Isolates No. | *E. faecium* | *S. aureus* | *P. aeruginosa* | *A. baumanii* | *K. pneumoniae* | *Enterobacter species* |
| AR7 | 12 | 15 | - | - | - | 10 |
| AR21 | 14 | - | - | 13 | 12 | 15 |
| AR42 | 11 | - | 13 | 15 | 14 | 14 |

### 3.2 Molecular Identification Results.

Strain identification was conducted using 16S rRNA analysis, and phylogenetic trees were constructed using the neighbor-joining tree method, as described by Idris et al. (2017). Through cultural, physiological, and molecular analysis, the AR7 strain was classified as belonging to the *Sphaerisporangium* species. On the other hand, the AR21 strain was found to be closely related to the *Micromonospora* species. Interestingly, isolate AR42 formed a distinct branch within a well-supported subclade that included the type strains of *Nonomuraea* species, Figure 1.

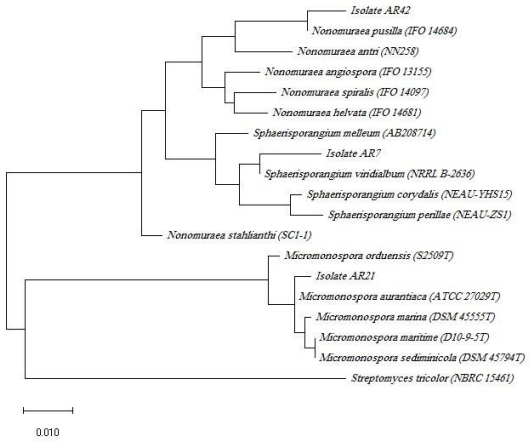


Figure 1. 16S rRNA gene neighbor-joining phylogenetic tree showing the relationships between isolates AR7, AR21, and AR42 and the different strains of more firmly related actinobacterial species.

### 3.3 Secondary Screening of Metabolite Extracts.

The crude extract obtained from the isolated AR7 strain exhibited antimicrobial activity against *E. faecium, Enterobacter species,* and *K. pneumoniae*. However, no activity was observed against *P. aeruginosa*, *M. luteus*, and *A. baumannii*. Conversely, the crude extract from the AR21 isolate demonstrated activity against *S. aureus* and *K. pneumoniae*. Additionally, the crude extract from the AR42 isolate showed inhibition zones against *K. pneumoniae* and *P. aeruginosa* (Table 2).

Table 2. Antimicrobial activity for actinobacterial metabolite extract against a panel of microorganisms.

| | Tested microorganisms | | | | | |
|---|---|---|---|---|---|---|
| Isolates No. | *E. faecium* | *S. aureus* | *P. aeruginosa* | *A. baumanii* | *K. pneumoniae* | *Enterobacter species* |
| AR7 | 18 | 20 | - | - | 12 | 10 |
| AR21 | 19 | - | - | 13 | 16 | 13 |
| AR42 | 12 | - | 15 | 12 | 14 | 13 |

### 3.4 HPLC-UV analysis

The methanol extract of isolate AR7 exhibited six peaks in the HPLC graph, with the first peak appearing at a retention time of 0.85 min and the last peak at a retention time of 3.78 min. Upon comparing these peaks with standards, it was determined that the fourth peak closely resembled the standard peak of Rifampin, which has a retention time of 1.401 min. Therefore, it can be inferred that sample AR7 is capable of producing Rifampin along with other compounds. Similarly, the methanol extract of isolate AR21 displayed eight peaks in the HPLC

graph, ranging from a retention time of 0.65 min to 3.92 min. Upon comparison with the standards, it was observed that the fourth peak closely resembled the standard peak of Rifampin. Thus, isolate AR21 shows potential as a producer of Rifampin. Furthermore, the methanol extract of isolate AR42 exhibited seven peaks in the HPLC graph within a retention time range of 0.81 min to 5.32 min (Figure 4). Upon comparison with the standard, it was observed that the fourth peak resembled the standard peak of Rifampin, indicating that isolate AR42 is a potential producer of Rifampin. Therefore, all three actinomycetes isolates, AR7, AR21, and AR42, are capable of producing Rifampin along with other antibiotic compounds (Table 3). In contrast, the remaining isolates did not exhibit peaks resembling the standard peak of Rifampin, suggesting that they do not produce Rifampin but may possess other bioactive compounds.

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Table 3. Retention time for Rifampin standard and methanol extracts by HPLC chromatogram.

| Samples | retention time | Area % |
|---|---|---|
| Rifampin | 1.401 | ------ |
| AA13 | 1.399 | 12.454 |
| AA12 | 1.408 | 9.794 |
| AA17 | 1.402 | 10.179 |

## 4. Conclusion.

This research has made significant contributions to our understanding of rare actinobacterial isolates sourced from desert soil. Notably, the methanol extracts of three potent actinobacterial isolates exhibited remarkable antimicrobial activity against various human microbial pathogens. It is particularly interesting that these rare actinobacterial species isolated from desert soil have demonstrated a high potential for producing rifampin. Specifically, the Sphaerisporangium species (AR7), Micromonospora species (AR21), and Nonomuraea species (AR42) showed detectable levels of rifampin through HPLC-UV analysis, where the retention times of the actinomycetes extracts aligned with the retention time of the rifampin standard. These findings highlight the need for further extensive investigations and large-scale isolation of bioactive compounds from these isolates, with the aim of exploring their potential applications against microbial pathogens.

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